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Ras recruits Raf-1 to the plasma membrane for activation by tyrosine phosphorylation

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A central feature of signal transduction downstream of both receptor and oncogenic tyrosine kinases is the Ras-dependent activation of a protein kinase cascade consisting of Raf-1, Mek (MAP kinase kinase) and ERKs (MAP kinases). To study the role of tyrosine kinase activity in the activation of Raf-1, we have examined the properties of p74Raf-1 and oncogenic Src that are necessary for activation of p74Raf-1. We show that in mammalian cells activation of p74Raf-1 by oncogenic Src requires pp60Src to be myristoylated and the ability of p74Raf-1 to interact with p21Ras-GTP. The Ras/Raf interaction is required for p21Ras-GTP to bring p74Raf-1 to the plasma membrane for phosphorylation at tyrosine 340 or 341, probably by membrane-bound pp60Src. When oncogenic Src is expressed with Raf-1, p74Raf-1 is activated 5-fold; however, when co-expressed with oncogenic Ras and Src, Raf-1 is activated 25-fold and this is associated with a further 3-fold increase in tyrosine phosphorylation. Thus, p21Ras-GTP is the limiting component in bringing p74Raf-1 to the plasma membrane for tyrosine phosphorylation. Using mutants of Raf-1 at Tyr340/341, we show that in addition to tyrosine phosphorylation at these sites, there is an additional activation step resulting from p21Ras-GTP recruiting p74Raf-1 to the plasma membrane. Thus, the role of Ras in Raf-1 activation is to bring p74Raf-1 to the plasma membrane for at least two different activation steps.

Key words: activation/plasma membrane/Raf-1/Ras/tyrosine phosphorylation

Introduction

Studies on the nematode worm *Caenorhabditis elegans*, the fly *Drosophila melanogaster* and on vertebrate cells have elucidated a conserved signalling cascade consisting of receptor tyrosine kinases, Ras guanine nucleotide binding proteins, the Raf-1 serine protein kinase, the dual specificity protein kinase MEK-1 (MAP kinase kinase) and extracellular signal regulated kinases ERKs (MAP kinases). The Ras/Raf/ERK signal transduction pathway appears to play a central role in cell behaviour since depending on cellular context it can control cell proliferation or differentiation (reviewed in Marshall, 1995). Activation of ERKs may regulate both transcription by the phosphorylation of transcription factors such as ELK-1 (Gille *et al.*, 1992; Janknecht *et al.*, 1993; Marais *et al.*, 1993) and protein synthesis through inactivation by phosphorylation of PHAS-1, an inhibitor of translational initiation (Haystead *et al.*, 1994; Pause *et al.*, 1994).

The mechanisms by which the ERKs and MEK-1 are activated by phosphorylation are now well understood (Payne *et al.*, 1991; Alessi *et al.*, 1994; Zheng and Guan, 1994); however, the mechanism of activation of p74Raf-1 appears more complex. The structure of p74Raf-1 contains three regions (CR1, CR2 and CR3) conserved among all Raf family kinases (Heidecker *et al.*, 1992). CR3 in the C-terminal half of the protein is the catalytic domain, while CR1 and CR2 comprise the N-terminal domain. CR1 contains a cysteine finger-like domain and the Ras binding domain (Vojtek *et al.*, 1993; Fabian *et al.*, 1994). The N-terminus is a negative regulatory domain because N-terminal truncation and fusion to retrovirus gag sequences generated the v-Raf oncogene with constitutive kinase activity. While p74Raf-1 binds directly to p21Ras-GTP, this binding does not activate the kinase activity of Raf-1 (Warne *et al.*, 1993; Zhang *et al.*, 1993), rather activation of Raf-1 by Ras requires Raf-1 and Ras to be co-expressed in cells reflecting the need for post-translational modification of p21Ras (Williams *et al.*, 1992; Fabian *et al.*, 1993; Kikuchi and Williams, 1994; Leevers *et al.*, 1994). The 14-3-3 family of proteins also appears to play a role in the activation of Raf-1 (Fantl *et al.*, 1994; Freed *et al.*, 1994; Fu *et al.*, 1994; Irie *et al.*, 1994). Co-expression of Raf-1 with oncogenic tyrosine kinases, such as v-Src, also activates Raf-1 and maximal activation of Raf-1 is obtained under conditions where both Ras and tyrosine kinases are active (Williams *et al.*, 1992; Fabian *et al.*, 1993, 1994). This activation is synergistic since the levels of Raf-1 activity that are obtained with oncogenic Ras plus v-Src are greater than the sum of the activations produced by oncogenic Ras or v-Src alone. The mechanism by which Ras and tyrosine kinases synergize or co-operate to maximally activate Raf-1 is not understood.

Experiments in which Raf-1 and oncogenic Src are overexpressed in insect cells suggest that Src and presumably other tyrosine kinases activate Raf-1 via direct phosphorylation on tyrosine residues 340 and 341 (Fabian *et al.*, 1993); however, tyrosine phosphorylation on these sites has not been reported in mammalian cells. The activation of Raf-1 by oncogenic Src through overexpression in insect cells also appears to be independent of p21Ras since a point mutant of Raf-1 (R89L) that destroys the interaction of Raf-1 with p21Ras and the activation of Raf-1 by p21Ras does not affect the ability of oncogenic Src to activate Raf-1 (Fabian *et al.*, 1994). These results have therefore been used to argue that the mechanisms of Raf-1 activation by Ras and oncogenic Src are different.

Recent work shows that the role of p21Ras in Raf-1

activation is for p21Ras-GTP to bring p74Raf-1 to the plasma membrane where it becomes activated. These conclusions are based on the findings that p74Raf-1 is associated with the plasma membrane in cells expressing oncogenic Ras (Traverse *et al.*, 1993; Leevers *et al.*, 1994; Wartmann and Davis, 1994), and that targeting Raf-1 to the plasma membrane by expressing Raf-1 constructs that contain Ras plasma membrane targeting signals activates the kinase activity of Raf-1 independently of Ras function (Leevers *et al.*, 1994; Stokoe *et al.*, 1994). This constitutive activation of Raf-1 following membrane targeting occurs in the absence of apparent growth factor signals, but membrane-targeted Raf-1 can be further activated by tyrosine kinase signalling. These results highlight the importance in mammalian cells of plasma membrane localization to the activation of Raf-1. Since synergistic stimulation of Raf-1 occurs when both tyrosine kinases and Ras are active, and as receptor tyrosine kinases and oncogenic tyrosine kinases like pp60Src are localized at the plasma membrane, we have investigated whether the synergy between Ras and Src results from Ras bringing Raf-1 to the plasma membrane in order for membrane-localized signalling events to take place. One potential signalling event from membrane-bound tyrosine kinases is tyrosine phosphorylation of Raf-1. Previous work has indicated a role for tyrosine phosphorylation in the activation of Raf-1 when it is expressed in insect cells with oncogenic Src (Fabian *et al.*, 1993). We have also investigated whether tyrosine phosphorylation is involved in the activation of Raf-1 when oncogenic Ras is expressed with Raf-1 or when Raf-1 is targeted to the plasma membrane.

Results

In mammalian cells activation of Raf-1 by oncogenic Src requires Ras–Raf interaction and membrane binding of Src

In order to investigate the requirements for Raf-1 activation in mammalian cells, we used a transient transfection system in NIH3T3 mouse fibroblasts. Epitope-tagged Raf-1 was co-expressed with activators oncogenic Ras, in which glycine 12 of Ha-Ras was replaced with the arginine substitution found in Harvey murine sarcoma virus (v-Ras) and oncogenic Src, where tyrosine 527 is replaced with phenylalanine (Y527FSrc; Cartwright *et al.*, 1987). Figure 1A shows that expression of the exogenous Raf-1 was readily detected by Western blotting for the myc epitope with the monoclonal antibody 9E10 (Evan *et al.*, 1985). As reported previously (Morrison *et al.*, 1988), activation of Raf-1 is associated with forms of Raf-1 that have a retarded electrophoretic mobility, although the significance of this reduced mobility, which is thought to reflect phosphorylation, is not clear (Samuels *et al.*, 1993). Some differences are apparent between the effects of Y527FSrc and v-Ras, and maximal retardation is found when Raf-1 is expressed together with the two activators.

The activity of Raf-1 in the cell lysates was determined in a coupled 'pull down' assay in which Raf-1 first phosphorylates and activates GST-Mek-1, and then the activated GST-Mek-1 phosphorylates and activates GST-ERK2 (Alessi *et al.*, 1994, 1995). The kinase activity of the GST-ERK2 is measured by its ability to phosphorylate myelin basic protein. Figure 1B shows that under the conditions we used, transfection of Y527FSrc with Raf-1 activates Raf-1 5-fold, while transfection with v-Ras activates Raf-1 weakly, with the range of activation over a number of experiments being between 1- and 2-fold. Co-transfection of Y527FSrc and v-Ras with Raf-1 activated Raf-1 25-fold, confirming that the synergy between Src and Ras in Raf-1 activation is not only found in insect cells, but also in mammalian cells.

Through the use of the Arg89Leu (R89LRaf-1) mutation in the Ras binding domain of Raf-1, which blocks the Ras/Raf interaction, Fabian *et al.* (1994) have shown that when expressed in insect cells, oncogenic Src will activate Raf-1 independently of Ras. In baculovirus infection of insect Sf9 cells, protein expression is very high and could lead to interactions between co-expressed proteins which do not occur at lower levels of expression. We therefore investigated in mammalian cells whether oncogenic Src could activate Raf-1 in the absence of a functional Ras/Raf interaction. We have previously shown that the Ras/Raf interaction leads to p74Raf-1 becoming associated with the plasma membrane in cells transformed with oncogenic Ras (Traverse *et al.*, 1993; Leevers *et al.*, 1994). Figure 1C shows that when expressed in NIH3T3 mouse fibroblasts, R89LRaf-1 fails to be translocated to the plasma membrane by co-expression with v-Ras. In contrast to the results with insect cells, co-expression with oncogenic Src does not activate R89LRaf-1 in mammalian cells (Figure 1B). As expected in mammalian cells, there is also no synergistic activation of R89LRaf-1 by co-expression with v-Ras and oncogenic Src. Leevers *et al.* (1994) and Stokoe *et al.* (1994) showed that the requirement for Ras in the activation of Raf-1 can be overcome by targeting Raf-1 to the plasma membrane by a C-terminal Ras membrane localization motif which consists of the last 20 amino acids of K-Ras4B (6KCAAX); we therefore investigated whether addition of such a motif to R89LRaf-1 would rescue its ability to be activated by Y527FSrc. Figure 1B shows that R89LRaf6KCAAX is activated by co-expression with Y527FSrc, demonstrating that the requirement for the Ras/Raf interaction for activation of Raf-1 by Y527FSrc is for p21Ras-GTP to translocate p74Raf-1 to the plasma membrane.

Myristoylation of pp60Src at Gly2 is essential for membrane localization and mutants that cannot be myristoylated (e.g. Gly2Ala) are transformation defective, but do not appear to be compromised in tyrosine kinase activity (Buss and Sefton, 1985). Since it is not clear which of the signal transduction pathways activated by oncogenic Src require Src to be membrane localized, we investigated whether myristoylation of Y527FSrc is required for it to activate Raf-1. Figure 2A shows that Y527FSrcG2A fails to activate Raf-1, furthermore there is no co-operation between Y527FSrcG2A and v-Ras in Raf-1 activation. In contrast, point mutants of Y527FSrc that compromise the SH2 domain (R175L), or the SH3 domain (W118A) (Mayer and Baltimore, 1993; Superti-Furga *et al.*, 1993), have little effect on the ability of Y527FSrc to activate Raf-1, or the ability of Y527FSrc to co-operate with v-Ras. These results show that for Src to activate Raf-1 in mammalian cells it has to be localized at the plasma membrane, and that SH2 and SH3 domain interactions of Src do not appear to be required.

These experiments therefore demonstrate that in mam-

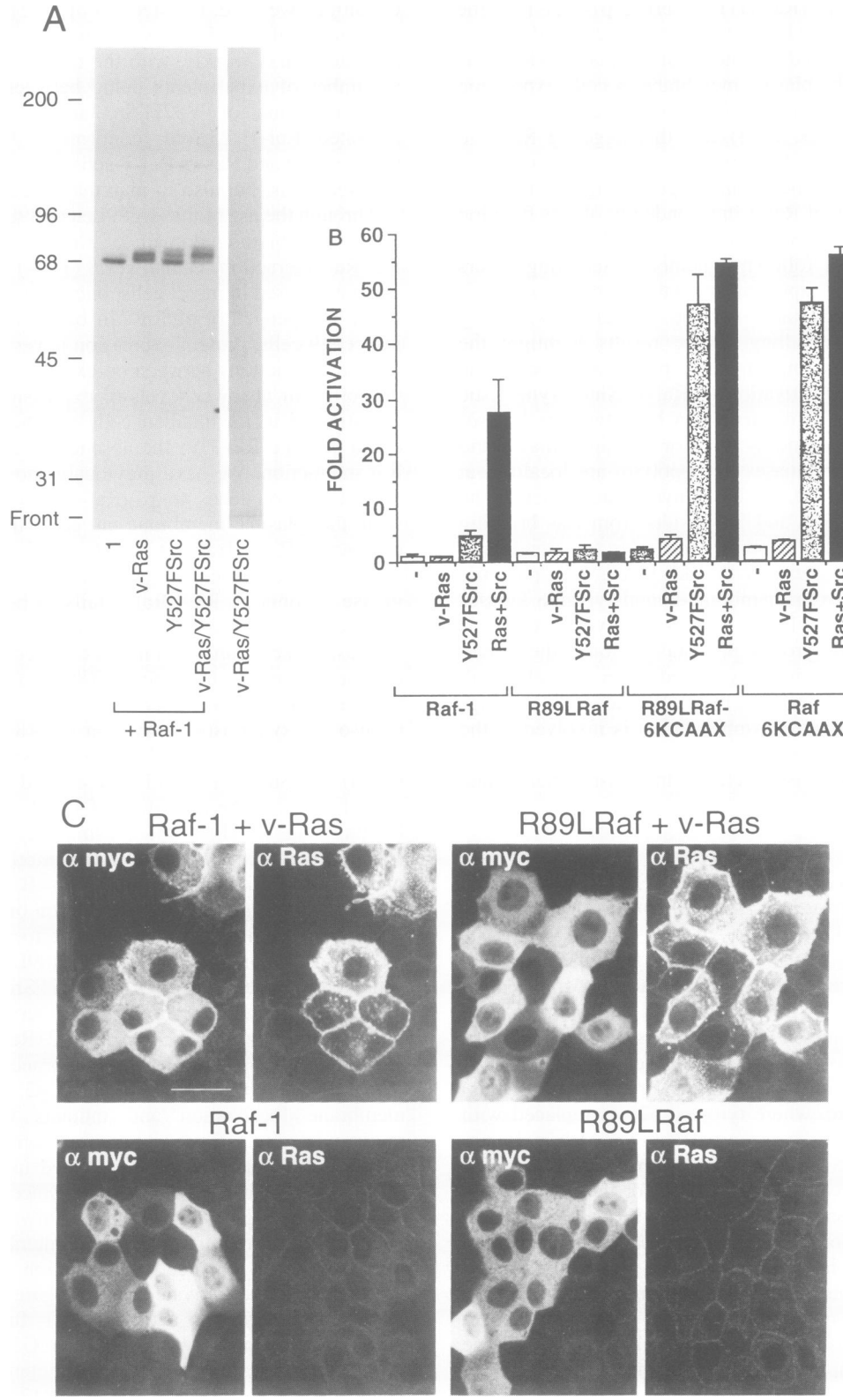

Fig. 1. The Ras/Raf interaction is required for activation of Raf-1 by oncogenic Src in mammalian cells. (**A**) Western blot of exogenous Raf-1 expressed on its own and with activators in NIH3T3 cell using transient transfection with lipofectAMINE reagent (Gibco/BRL). Cell extract (45 μl) were electrophoresed in 7% SDS gels, transferred to nitrocellulose and detected using the 9E10 monoclonal antibody. (**B**) Raf-1 activity assay. Raf-1 constructs, v-Ras and Y527FSrc were expressed in NIH3T3 cells and assayed for kinase activity as described in Materials and methods. (**C**) R89LRaf-1 fails to be translocated to the plasma membrane by p21Ras-GTP. MDCK cells were microinjected with Raf-1 or R89LRaf-1 alone (lower panels) or in combination with v-Ras (upper panels). Cells were stained for exogenous myc-tagged Raf with 9E10 and for Ras with a mixture of Y13-238 and Y13-259.

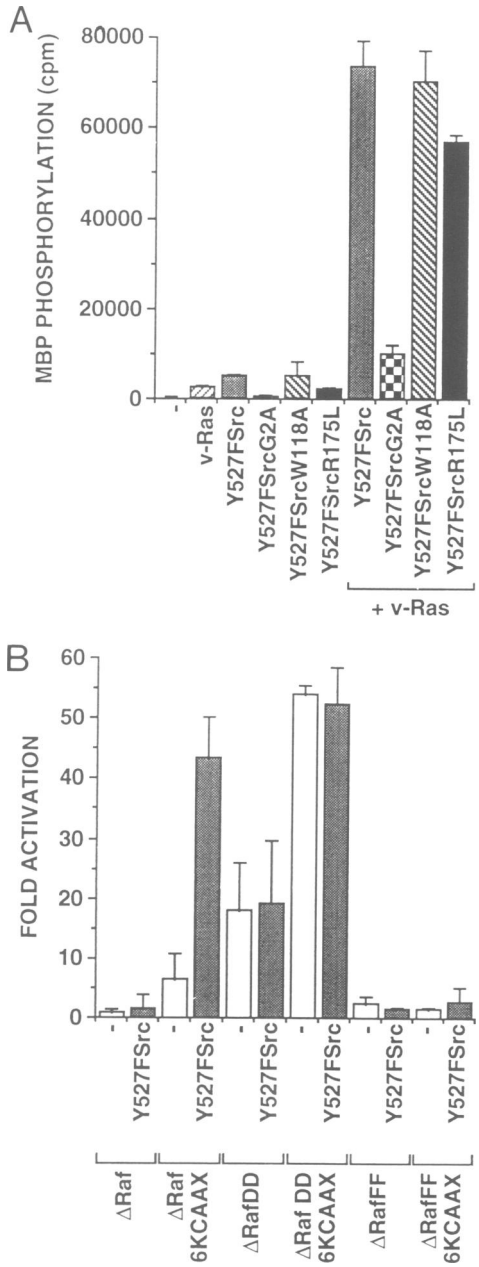

Fig. 2. Activation of Raf by oncogenic Src requires membrane localization of Src. (**A**) Src needs to be myristoylated in order to activate Raf-1. Raf-1 was expressed in NIH3T3 cells in combination with v-Ras and with Y527FSrc, or with Y527FSrc mutants in the myristoylation signal (Y527FSrcG2A), or an inactivating mutation in the SH3 domain (Y527FSrcW118A), or an inactivating mutation in the SH2 domain (Y527FSrcR175L). The levels of expression of each Src mutant were similar to each other, as determined by Western blotting analysis (data not shown). p74Raf-1 levels were normalized to each other and the kinase activities determined in a coupled pull down assay with GST-Mek-1 and GST-ERK2. (**B**) Effect of plasma membrane targeting of the ΔRaf catalytic domain on activation by Y527FSrc. ΔRaf constructs based on the catalytic domain of Raf (amino acids 325–648) were expressed with and without Y527FSrc, and their kinase activity measured. ΔRaf6KCAAX consists of Raf-1 amino acids 325–648 fused to the plasma membrane localization signals of K-Ras4B, ΔRafDD has tyrosine 340 and 341 replaced by aspartic acid, ΔRafDD6KCAAX is ΔRafDD fused to the plasma membrane localization signals of K-Ras4B, ΔRafFF has tyrosine 340 and 341 replaced by phenylalanine and ΔRafFF6KCAAX comprises ΔRafFF fused to the plasma membrane localization signals of K-Ras4B.

malian cells a functional Ras/Raf interaction is required for Y527FSrc-mediated activation of Raf-1 and that both Raf-1 and Y527FSrc must be localized at the plasma membrane. We believe that the observation that activation of Raf-1 by oncogenic Src appears to be independent of the Ras/Raf interaction when the proteins are expressed in insect cells is a consequence of the very high levels of protein expression in this system. We have previously shown that a Raf protein that consists of the catalytic domain amino acids 325–648, ΔRaf (Howe *et al.*, 1992), is cytosolic even in cells expressing oncogenic Ras because it lacks the N-terminal Ras binding domain (Traverse *et al.*, 1993). In COS cells, expression of such a protein leads to the activation of Erk2 (Howe *et al.*, 1992); however, when expressed in NIH3T3, Δ-Raf produces little activation of Gst-Mek-1 (Figure 2B), presumably as a result of the lower levels of expression, or basal tyrosine kinase activity in these cells. We therefore investigated whether Δ-Raf could be activated by co-expression with Y527FSrc in NIH3T3 cells. Figure 2B shows that co-expression of Δ-Raf with Y527FSrc does not affect the activity of Δ-Raf; however, when Δ-Raf is fused to the 6KCAAX motif to target it to the plasma membrane, its activity is increased 5-fold and it is further stimulated 7-fold by co-expression with Y527FSrc. This maps an effect of Y527FSrc to the catalytic domain of Raf-1 which is independent of the ability of oncogenic Src to promote the formation of p21Ras-GTP.

Ras recruits Raf-1 to the plasma membrane for tyrosine phosphorylation

Co-infection of insect cells with baculoviruses expressing Y527FSrc and Raf-1 leads to tyrosine phosphorylation of Raf-1 (Fabian *et al.*, 1993), and activation by a Ras-independent route (Fabian *et al.*, 1994). As we have shown above, in mammalian cells Y527FSrc activation does require an intact Ras/Raf interaction, suggesting that Y527FSrc could activate Raf-1 by a different mechanism in the insect cell overexpression system compared with mammalian cells. Since cells transformed by v-Src are known to contain elevated levels of p21Ras-GTP (Gibbs *et al.*, 1990), one possibility is that activation of Raf-1 by oncogenic Src in mammalian cells is solely mediated by oncogenic Src activating Ras guanine nucleotide mechanisms. Such a mechanism is consistent with our demonstration that Y527FSrc activation of Raf-1 in mammalian cells is critically dependent on the Ras/Raf interaction. In the expression system we use, Y527FSrc activates Raf-1 more strongly than v-Ras, but only in v-Ras expressing cells is there pronounced association of Raf-1 with the plasma membrane (data not shown). Further, the fact that there is synergy between oncogenic Src and oncogenic Ras in the activation of Raf-1 in mammalian cells suggests that elevation of p21Ras-GTP is only part of the mechanism of Raf-1 activation by Y527FSrc. Finally, we have shown that when the catalytic domain of Raf-1 is targeted to the plasma membrane (ΔRaf6KCAAX) it is further activated by Y527FSrc, demonstrating that the activation of Raf-1 by Y527FSrc in mammalian cells involves a step in addition to Ras-mediated recruitment to the plasma membrane. These observations led us to investigate whether tyrosine phosphorylation is involved in the activation of Raf-1 in mammalian cells.

Table I. % Phosphotyrosine on Raf-1

	Empty vector		+v-Ras		+Y527FSrc		+v-Ras/Y527FSrc		*n*
	%P-Y	Range	%P-Y	Range	%P-Y	Range	%P-Y	Range	
Raf-1	0.9	0.7–1.4	0.5	0.3–0.7	1.9	1.7–2.5	6.0	4.5–7.9	(5)
R89LRaf	1.3	0.6–1.8	0.6	0.6	1.4	1.3–1.5	0.8	0.6–1.0	(3)
R89LRafCAAX	0.7	0.5–1.1	nd	nd	2.8	1.9–3.5	nd	nd	(3)

The radioactivity in phosphothreonine, phosphoserine and phosphotyrosine was estimated using a PhosphorImager and the percent phosphotyrosine expressed as percent of total phosphoamino acids. *n* = number of separate experiments.

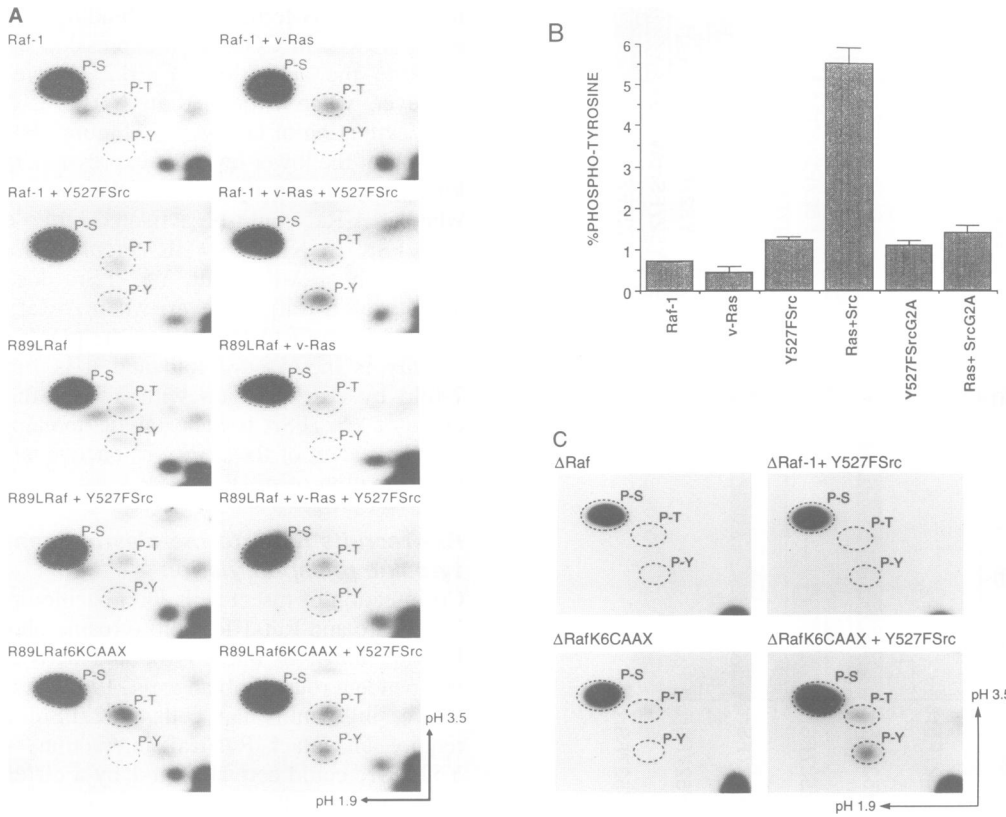

Fig. 3. Phosphoamino acid analysis of Raf-1 co-expressed with v-Ras and Y527FSrc. (**A**) Ras/Raf interaction is required for Y527FSrc-mediated phosphorylation of Raf-1. Raf-1, R89LRaf or R89LRaf6KCAAX were transiently expressed in NIH3T3 cells on their own, with v-Ras, Y527FSrc or v-Ras plus Y527FSrc and labelled with [^{32}P]orthophosphate. The myc epitope-tagged Raf proteins were immunoprecipated, electophoresed and transferred to PVDF membrane. A total of 1000 c.p.m. of each sample was then subjected to phosphoamino acid analysis; the position of each ^{32}P-labelled phosphoamino acid was identified by reference to the ninhydrin-stained standards. (**B**) Myristoylation of Y527FSrc is required for tyrosine phosphorylation of Raf-1. Raf-1 was expressed on its own or co-expressed with either wild-type Y527FSrc, wild-type Y527FSrc plus v-Ras, Y527FSrcG2A or Y527FSrcG2A plus v-Ras and ^{32}P-labelled proteins subjected to phosphoamino acid analysis. The results are expressed as % phospho-tyrosine as a proportion of the total amount of phosphoamino acids. (**C**) Tyrosine phosphorylation of the ΔRaf catalytic domain requires plasma membrane targeting. ΔRaf or ΔRaf6KCAAX were expressed on their own or with Y527FSrc and ^{32}P-labelled proteins subjected to phosphoamino acid analysis.

NIH3T3 cells transfected with either Raf-1 alone, Raf-1 with v-Ras or Y527FSrc, or Raf-1 with v-Ras and Y527FSrc were metabolically labelled with [^{32}P]orthophosphate and Raf-1 proteins immunoprecipitated using the 9E10 monoclonal antibody against the myc epitope and subjected to phosphoamino acid analysis. Table I and Figure 3A show that there is a 6.6-fold increase in the amount of phosphotyrosine on p74Raf-1, when it is co-expressed with v-Ras and Y527FSrc compared with a 2-fold increase when Raf-1 is expressed with Y527FSrc alone. These results in mammalian cells differ markedly from those obtained in insect cells where co-expression of v-Ras with oncogenic Src did not increase Src-promoted tyrosine phosphorylation of p74Raf-1 (Fabian *et al.*, 1993). These experiments show that the maximal activation of Raf-1 on co-expression with v-Ras and Y527FSrc is associated with the maximal increase in the level of phosphotyrosine. Note that when expressed with v-Ras alone the per cent phosphotyrosine on Raf-1 is decreased to 0.5%, but it should be noted that there is 3–4 times more phosphorylation of p74Raf-1 in cells expressing v-Ras due to a large increase in the levels of phosphothreonine and phosphoserine; thus, the proportion of p74Raf-1 that is tyrosine phosphorylated is increased in v-Ras-expressing cells.

The requirement for the Ras/Raf interaction in order for oncogenic Src to activate Raf-1, which we demonstrated using the R89L mutant (see Figure 1A), led us to

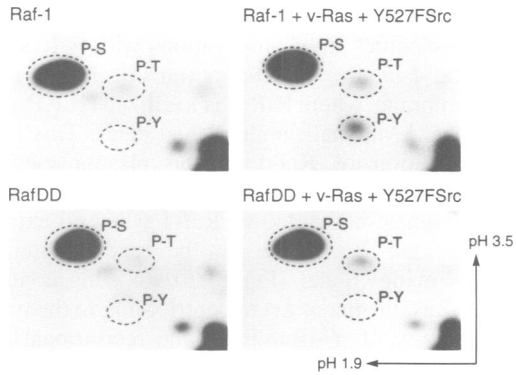

Fig. 4. Tyrosine residues 340 and 341 play a critical role in the activation of Raf-1 by Y527FSrc in mammalian cells. Mutation of tyrosine 340/341 to aspartic acid blocks Y527FSrc-mediated tyrosine phosphorylation of Raf-1. Raf-1 or RafY340DY341D were expressed on their own or together with v-Ras plus Y527FSrc and ^{32}P-labelled proteins subjected to phosphoamino acid analysis.

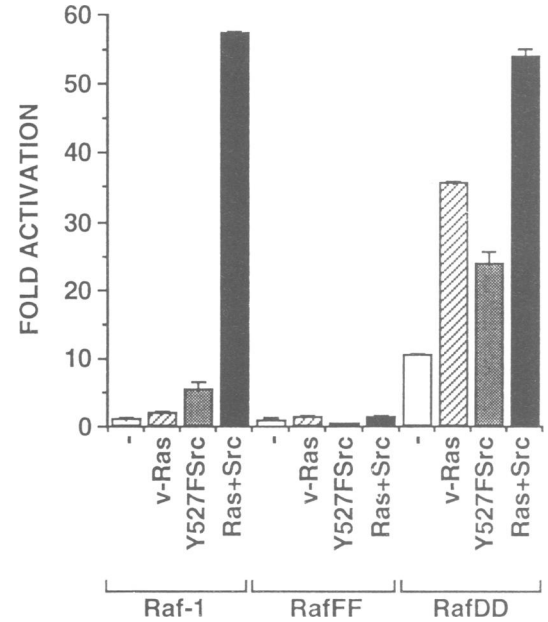

Fig. 5. Evidence for activation steps in addition to phosphorylation on tyrosine 340/341. Raf-1, RafY340FY341F, RafY340DY341D were expressed on their own, with v-Ras, Y527FSrc or with v-Ras plus Y527FSrc, and kinase activity measured in the coupled pull down assay.

investigate whether this interaction was required for tyrosine phosphorylation of Raf-1. In three separate experiments, the level of phosphotyrosine on R89LRaf-1 (1.3%, range 0.6–1.8%) was not increased when co-expressed with Y527FSrc (mean 1.4% phosphotyrosine, range 1.3–1.5%), see Figure 3A. However, the effect of the R89L mutation in blocking tyrosine phosphorylation could be overcome by directly targeting R89LRaf to the plasma membrane. Figure 3A shows that the level of phosphotyrosine on R89LRaf6KCAAX increases 5-fold when it is co-expressed with Y527FSrc.

The data presented in Figure 2A show that pp60Y527FSrc must be myristoylated in order to permit membrane localization and activation of Raf-1, we therefore investigated whether myristoylation was required for tyrosine phosphorylation of Raf-1. Figure 3B shows that expression of the mutant Y527FSrcG2A with Raf-1 fails to increase the level of phosphotyrosine on Raf-1, and that there is no co-operation between Y527FSrcG2A and v-Ras in promoting tyrosine phosphorylation of Raf-1. Thus, as for activation of Raf-1 by Y527FSrc, tyrosine phosphorylation requires both the Ras/Raf interaction and membrane localization of Y527FSrc.

These experiments show that in mammalian cells tyrosine phosphorylation of Raf-1 is correlated with the ability of Y527FSrc to activate Raf-1. This tyrosine phosphorylation requires the Ras/Raf interaction, arguing that p21Ras-GTP recruits p74Raf-1 to the plasma membrane for tyrosine phosphorylation. Figure 3C shows that the failure of Y527FSrc to activate the cytosolic ΔRaf results from the failure of ΔRaf to become tyrosine phosphorylated, but targeting ΔRaf to the plasma membrane as ΔRaf6KCAAX results in tyrosine phosphorylation (a 9-fold increase to 6.2% phosphotyrosine) and activation (see Figure 2B). This further reinforces the point that a role of membrane recruitment is to permit tyrosine phosphorylation.

Mapping of the tyrosine residues in Raf-1 that are phosphorylated by oncogenic Src when the two proteins are co-expressed in insect cells shows them to be Tyr 340 and Tyr 341 (Fabian *et al.*, 1993), we therefore investigated whether the same two residues were phosphorylated in mammalian cells. When Raf-1Y340FY340F was expressed with Y527FSrc and v-Ras, we found a smaller amount of phosphotyrosine on Raf-1 than with wild-type Raf-1 (2.4% versus 7.2%) and Raf-1Y340FY341F showed no activation when expressed with Y527FSrc (Figure 5), suggesting that Tyr 340 and Tyr 341 are the sites on Raf-1 responsible for activation by Y527FSrc. However, the failure to activate Raf-1Y340FY341F could be a result of an adverse effect of the two phenylalanine substitutions on the conformation of Raf-1. We therefore exploited the observation that if Tyr340 and Tyr341 are replaced with aspartic acid, the resulting protein is constitutively active (Fabian *et al.*, 1993). Figure 4 shows that the level of tyrosine phosphorylation on Raf-1Y340DY341D increases to only 1.2% when co-expressed with Y527FSrc and v-Ras, compared with an increase of 7.2% on wild-type Raf-1. These results therefore argue that Tyr340/341 are major sites of phosphorylation on Raf-1 which contribute to activation of kinase activity against Mek-1, although the small increase in phosphotyrosine on Raf-1Y340FY341F when it is co-expressed with v-Ras and Y527FSrc suggests that there may be some additional phosphorylation sites.

In addition to phosphorylation on Tyr 340 and Tyr 341 there are additional mechanisms for activation of Raf-1

An interpretation of the experiments reported here is that the critical event in the activation of Raf-1 is phosphorylation of Tyr340 and 341. The role of the Ras/Raf interaction would then be to bring p74Raf-1 to the plasma membrane for tyrosine phosphorylation at these sites. In this model, the activation of Raf-1 when expressed with v-Ras alone would result from basal tyrosine kinase activity in the transfected cells. However, examination of the response of Raf-1Y340DY341D to v-Ras and Y527FSrc argues that tyrosine phosphorylation at Tyr 340/341 is not the only mechanism that results in activation. Significantly,

Raf-1Y340DY341D is 3.5-fold further activated by v-Ras (see Figure 5), suggesting that there is a Ras-dependent activation step which is separable from tyrosine phosphorylation at 340/341. If tyrosine phosphorylation at 340/341 were the sole mechanism of activation, then co-expression with v-Ras would not be expected to affect the activity of Raf-1Y340DY341D. Co-expression of Raf-1Y340DY341D with Y527FSrc also leads to an increase in activity (2.5-fold), but in contrast to wild-type Raf-1, with Raf-1Y340DY341D Y527FSrc produces a smaller activation than v-Ras. In order to determine whether the further activation of Raf-1Y340DY341D by Ras results from p21Ras-GTP recruiting it to the plasma membrane or some other effect of Ras, we made use of constructs containing the isolated catalytic domain (ΔRaf) and Ras plasma membrane targeting signals. The activity of ΔRafDD, in which Tyr340 and Tyr341 in the isolated catalytic domain are replaced by aspartic acid residues, is 18-fold higher than the ΔRaf catalytic domain alone (Figure 2B), consistent with the important role of Tyr340/341 in activation, but Figure 2B shows that if ΔRafDD is targeted to the plasma membrane (ΔRafDDCAAX) its activity is even higher (54-fold). Notably, the activity of ΔRafDDCAAX is unaffected by co-expression with Y527FSrc, arguing that the additional activation produced by bringing ΔRafDD to the plasma membrane is not a consequence of further tyrosine phosphorylation. These results imply that the further activation of Raf-1Y340DY341D by Y527FSrc could result solely from Y527FSrc promoting the formation of p21Ras-GTP, rather than the low level of tyrosine phosphorylation on this protein.

Discussion

We have shown that in mammalian cells the activation of Raf-1 by co-expression with Y527FSrc is critically dependent on the ability of p74Raf-1 to interact with p21Ras-GTP and on pp60Y527FSrc to be myristoylated. The dependence on the Ras/Raf interaction is not a consequence of the sole mechanism of Y527FSrc activation of Raf-1 being to raise the level of p21Ras-GTP in cells, but rather reflects the requirement of the interaction with p21Ras-GTP to bring p74Raf-1 to the plasma membrane for phosphorylation by membrane-bound tyrosine kinases. Although there is no formal demonstration that pp60Y527FSrc directly phosphorylates p74Raf-1, the simplest interpretation of these experiments and others in insect cells (Fabian *et al.*, 1993) is that p74Raf-1 is a substrate of Src although the amino acid sequence around Tyr340/341 does not fit particularly well the preferred substrate recognition sites identified with degenerate peptide libraries (Songyang *et al.*, 1995).

Previous experiments (Traverse *et al.*, 1993; Leevers *et al.*, 1994; Stokoe *et al.*, 1994) and those presented here reinforce the importance of the Ras/Raf-1 interaction for Raf-1 activation. The simplest interpretation of these experiments is that direct Ras/Raf-1 binding is responsible solely for translocating Raf-1 to the plasma membrane. However, it cannot be formally ruled out that the role of direct Ras/Raf-1 binding is the first step in bringing Raf-1 to the plasma membrane, where an additional event (perhaps a Ras-GTP-induced conformation change) allows Raf-1 to become held in the plasma membrane. Support for this view comes from observations with R-Ras which can bind Raf-1 *in vitro*, but cannot translocate Raf-1 to the plasma membrane, where R-Ras is localized (H.F.Paterson, A.Self and C.J.Marshall, unpublished data). This implies that translocation of Raf-1 to the plasma membrane requires more than the direct binding of Raf-1 to Ras.

The synergistic activation of Raf-1 when co-expressed with v-Ras and Y527FSrc can be readily interpreted on the following model (Figure 6). In cells containing oncogenic Ras, the major event contributing to the tyrosine phosphorylation of p74Raf-1 will be recruitment to the plasma membrane of Raf-1, where it will be phosphorylated by the low level of basal tyrosine kinase activity (Figure 6B). In cells containing Y527FSrc, there is both a Y527FSrc-mediated increase in the level of p21Ras-GTP (Gibbs *et al.*, 1990) to recruit p74Raf-1 to the plasma membrane and increased tyrosine kinase activity to phosphorylate the membrane-localized p74Raf-1. Unlike the situation with oncogenic Ras, however, the increase in the level of p21Ras-GTP resulting from Y527FSrc expression will be controlled by the Ras GTPase activating proteins p120RasGAP and Neurofibromin (Boguski and McCormick, 1993). Indeed, in Y527FSrc-expressing cells we do not see a wholesale recruitment of p74Raf-1 to the plasma membrane (data not shown) so membrane recruitment is likely to be the limiting step (Figure 6C). Where Y527FSrc and oncogenic Ras are co-expressed, neither membrane recruitment nor tyrosine kinase activity will be limiting so that maximal tyrosine phosphorylation results (Figure 6D).

These experiments also show that there is another component to the activation of Raf-1. With phenylalanine or aspartic acid substitutions at tyrosine 340/341, we can show that tyrosine phosphorylation at these sites is one mechanism that leads to activation, but there is an additional mechanism(s). This additional mechanism also involves p21Ras-GTP recruiting p74Raf-1 to the plasma membrane, but its nature is unclear. Given that cytosolic p74Raf-1 is already complexed to 14-3-3 proteins (Freed *et al.*, 1994), it is unlikely that the unknown step is to bring p74Raf-1 to the membrane to interact with 14-3-3. The maximal level of activation of Raf-1 we observe when it is co-expressed with oncogenic Ras and oncogenic Src, therefore, is the combination of the synergy between Ras and Src in promoting tyrosine phosphorylation of p74Raf-1 and another membrane-bound activation step.

The idea that there must be additional membrane-localized events that can activate Raf-1 is supported by examining the sequences of other members of the Raf family and Raf proteins in other species. Mammalian B-Raf has aspartic acid residues at the positions equivalent to tyrosine 340/341(Sithanandam *et al.*, 1990) in Raf-1 and is activated by co-expression with v-Ras (R.Marais and C.J.Marshall, unpublished results). Further, the Raf proteins of *D.melanogaster* and *C.elegans*, which genetic analysis shows are dependent on Ras for their activation, also have aspartic or glutamic acid at the position equivalent to 340 and do not have a tyrosine residue at 341 (Mark *et al.*, 1987; Han *et al.*, 1993). Thus, a negative charge at 340 is an important component of the kinase activity of Raf proteins and this negative charge can either be achieved by an acidic amino acid or by phosphorylation

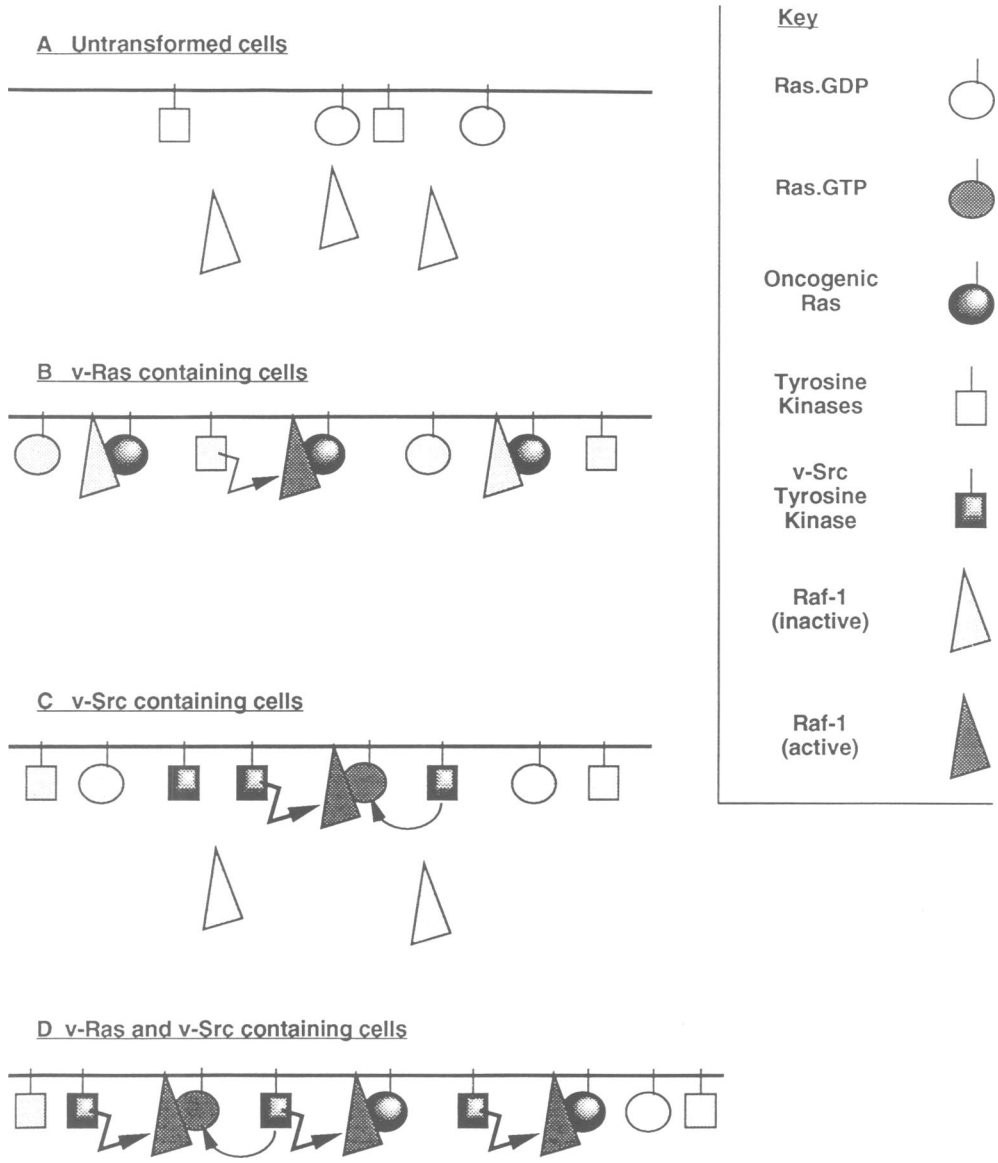

Fig. 6. Model to illustrate how oncogenic Ras and Src synergize to promote tyrosine phosphorylation of Raf and activation via p21Ras-GTP recruiting p74Raf-1 to the plasma membrane for phosphorylation by membrane-bound tyrosine kinases.

of tyrosine. In vertebrate cells, the phosphorylation of tyrosine appears to be an additional level of control imposed on Raf-1 activation. As a consequence of this additional level of control, oncogenic Ras and a tyrosine kinase such as Src can synergize to produce maximal Raf-1 activation.

These experiments point to tyrosine phosphorylation of p74Raf-1 being an important component of transformation by oncogenic tyrosine kinases. We have shown previously that signalling through the MAP kinase pathway is necessary for transformation by v-Src since expression of interfering negative mutants of Mek-1 blocks transformation by v-Src (Cowley *et al.*, 1994). The importance of tyrosine phosphorylation in the activation of Raf-1 by growth factor signalling rather than by an oncogenic tyrosine kinase is still unclear. Low levels of phosphotyrosine have been reported on p74Raf-1 following PDGF treatment of 3T3 fibroblasts (Morrison *et al.*, 1989) and interleukin-2 (IL2) treatment of T cells (Turner *et al.*, 1993). However, we have been unable to detect phosphotyrosine on p74Raf-1 following EGF treatment of Swiss 3T3 cells under conditions where p74Raf-1 is 30- to 40-fold activated. Thus, for this stimulus the major route to activation of p74Raf-1 following receptor tyrosine kinase activation may be via the formation of p21Ras-GTP and the unknown step.

Materials and methods

Cell culture and transfection

The cDNAs for human Raf-1, oncogenic Src and human c-Src in which tyrosine 527 was replaced with phenylalanine (Cartwright *et al.*, 1987), and v-Ras, which contains arginine to glycine substitution at codon 12, were cloned into the plasmid pEFPlink.2 (R.Marais, unpublished), a mammalian expression vector derived from pEFBos (Mizushima and Nagata, 1990) that uses the elongation factor 1a promoter to direct high levels of expression of cloned cDNAs. The ΔRaf construct contains amino acids 325–648 of Raf-1 (Howe *et al.*, 1992). The myc epitope tag (GGEQKLISEEDL) recognized by monoclonal antibody 9E10 (Evan

et al., 1985) was incorporated at the N-terminus of all Raf constructs. For transfection with lipofectAMINE (Gibco-Life Sciences), 1.5×10^5 NIH3T3 cells were plated into 30 mm tissue culture dishes and incubated overnight in 10% fetal calf serum (FCS)/Dulbecco's modified Eagle's medium (DMEM). The cells were washed with serum-free medium and DNA:lipofectAMINE (0.4 μg:6 μl) complexes added to the cells in 1 ml serum-free medium. The cells were incubated for 6 h in the presence of the DNA:lipofectAMINE complexes, and then washed and incubated in 10% FCS/DMEM for 48 h.

Raf-1 kinase assays

All extraction procedures were performed at 4°C. Cells were washed twice with phosphate buffered saline (PBSA) and extracted in 40 μl of extraction buffer [20 mM Tris–HCl (pH 7.5), 1 mM EDTA, 1.5 M KCl, 5% v/v glycerol, 1% v/v Triton X-100, 0.3% v/v 2-mercaptoethanol, 5 mM NaF, 0.2 mM Na_3VO_4, 1 μM microcystin LR, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 1 mM benzamidine, 1 mM p-aminoethyl-benzene sulfonyl fluoride]. The extract was diluted with 160 μl dilution buffer (prepared as for extraction buffer, but without any KCl and containing 10% v/v glycerol), the DNA was sheared and the extract centrifuged at 13 000 r.p.m. in a microfuge. The supernatant was removed and stored at −70°C. The amount of Raf-1 protein in the extracts was normalized by Western blotting with 9E10, using [^{125}I]protein A as the detection method with quantitation by PhosphorImager analysis (Molecular Dynamics). For Raf-1 protein kinase assay, equal amounts of Raf-1 protein standardized by Western blotting were used in a 'pull-down' kinase assay using GST-Mek-1 (Alessi *et al.*, 1994, 1995). Cell extract in a total volume of 3 μl was mixed with 20 μl reactivation buffer [30 mM Tris (pH 7.5), 0.1 mM EGTA, 0.3% v/v 2-mercaptoethanol, 0.03% Brij35, 10 mM $MgCl_2$, 0.1% Triton X-100, 5 mM NaF, 0.2 mM Na_3VO_4, 0.1 μM microcystin LR, 0.8 mM ATP] containing 6.5 μg/ml GST-Mek-1 (Alessi *et al.*, 1994). After incubation for 30 min at 30°C, the activation of GST-Mek-1 was terminated by the addition of 20 μl of buffer C [50 mM Tris (pH 7.5), 0.03% Brij35, 0.1 mM EGTA, 0.1% v/v 2-mercaptoethanol] containing 20 mM EDTA and 5 μl of glutathione–Sepharose (Pharmacia). The reactions were then shaken for 20–30 min at 4°C to capture the GST-Mek-1, washed twice with buffer C and then the GST-Mek-1 bound to glutathione–Sepharose incubated with 20 μl reactivation buffer containing 20 mM glutathionine and 100 μg/ml GST-ERK2 (Stokoe *et al.*, 1992). After 30 min at 30°C, the activation of GST-ERK2 was stopped with 2 μl of 120 mM EDTA, and 3 μl taken and incubated with 47 μl 50 mM Tris (pH 7.5), 0.1 mM EGTA, 0.4 mg/ml myelin basic protein, 12.5 mM $MgCl_2$, 0.125 mM [γ-^{32}P]ATP (10^6 c.p.m./nmol) to assay for myelin basic protein kinase activity. After 10 min at 30°C, the reactions were terminated by spotting 40 μl onto squares of P81 paper which were washed four times in 75 mM phosphoric acid and then counted by Cerenkov counting. Control experiments show that this assay is dependent on the presence of the transfected Raf-1: without exogenous Raf, the phosphorylation of MBP obtained when v-Ras and Y527FSrc were transfected together was <3% of that obtained when Raf-1, v-Ras and Y527FSrc are co-expressed. After subtraction of background radioactivity, estimated by blank assays set up with lysis buffer only, fold activations were determined by dividing the radioactivity in MBP for Raf with activators by the radioactivity in MBP for Raf without activators. Each determination was carried out in triplicate.

Phosphoamino acid analysis

NIH3T3 cells were transfected with expression plasmids as indicated. Forty hours after the transfection, the cells were placed into 1 ml phosphate-free medium with 20% dialysed FCS and 1 mCi [^{32}P]orthophosphate (Amersham PBS11). The cells were incubated for a further 6 h and extracted as described above. The Raf-1 was immunoprecipitated with 10 μg 9E10 monoclonal antibody coupled to protein G–Sepharose as described (Leevers *et al.*, 1994). The immunoprecipitated proteins were resolved in 7% SDS gels, transferred to Immobilon P (Millipore) membranes and the phosphorylated Raf-1 bands were excised and phosphoamino acid analysis performed as described (Kamps, 1991). Quantification of radioactivity incorporated into phosphoamino acids was carried out using a PhosphorImager.

Microinjection and immunofluorescence analysis

Microinjections were performed on a Zeiss Microinjection Workstation (Carl Zeiss, Oberkochen). Areas of cells to be injected were delineated with a scalpel blade on the tissue culture surface of 60 mm Petri dishes. All plasmids were injected into the nucleus of target cells at a concentration of 50 μg/ml. After overnight culture, cells were fixed in 4% formaldehyde and permeabilized in 0.2% Triton X-100 (15 min). After quenching in 100 mM glycine (15 min) and 10% FCS (15 min), cells were incubated for 1 h in ascitic fluid diluted 1:200 containing a mixture of mouse monoclonal antibody 9E10 (for the myc epitope) and rat monoclonal antibodies Y13-238 plus Y13-259 to visualize p21Ras (Furth *et al.*, 1982), then fluorescein isothiocyanate (FITC)–anti-mouse and Texas Red–anti-rat fluorescent antibody conjugates (1 h). Labelled preparations were mounted under glass coverslips, and examined with a Biorad MRC 600 confocal imaging system in conjunction with a Nikon Diaphot epifluorescence microscope.

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